



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (51) International Patent Classification: C12P 7/00, C12P 39/00 | A1 | (11) International Publication Number: WO 00/56911 (43) International Publication Date: 28 September 2000 (28.09.2000) |
| (21) International Application Number: PCT/US00/06808 | | Published |
| (22) International Filing Date: 16 March 2000 (16.03.2000) | | |
| (30) Priority Data: 09/274,732 23 March 1999 (23.03.1999) US | | |
| (60) Parent Application or Grant BOARD OF TRUSTEES OPERATING MICHIGAN STATE UNIVERSITY [/]; O. FROST, John, W. [/]; O. HANSEN, Chad, A. [/]; O. FROST, John, W. [/]; O. HANSEN, Chad, A. [/]; O. GIBBS, Barbara, S. ; O. | | |
| <p>(54) Title: SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1,2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1-PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DÉHYDROGENASE</p> <p>(54) Titre: SYNTHESE DE 1,2,3,4-TETRAHYDROXYBENZENES ET DE 1,2,3-TRIHYDROXYBENZENES AU MOYEN DE MYO-INOSITOL-1PHOSPHATE SYNTHASE ET DE MYO-INOSITOL 2-DEHYDROGENASE.</p> <p>(57) Abstract A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided. Methods of producing 1,2,3,4-tetrahydroxybenzene acid from a carbon source based on the synthesis scheme are also provided. Methods are also provided for converting 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene by catalytic hydrogenation.</p> <p>(57) Abrégé L'invention porte sur un processus de synthèse par génie biologique de 1,2,3,4-tétrahydroxybenzène à partir d'une source de carbone, sur des procédés de production d'acide 1,2,3,4- tétrahydroxybenzène à partir d'une source de carbone au moyen du susdit processus, et sur des procédés de conversion du 1,2,3,4- tétrahydroxybenzène en 1,2,3-trihydroxybenzène par hydrogénéation catalytique.</p> | | |

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| <p>(54) Title: SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1,2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1-PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DEHYDROGENASE</p> <p style="text-align: center;"> Key: (a) phosphoenolpyruvate:carbohydrate phosphotransferase; (b) myo-inositol 1-phosphate synthase; (c) phosphatase activity; (d) dehydrogenase activity; (e) 0.5 M H₂SO₄, H₂O, reflux. </p> <p>(57) Abstract</p> <p>A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided. Methods of producing 1,2,3,4-tetrahydroxybenzene acid from a carbon source based on the synthesis scheme are also provided. Methods are also provided for converting 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene by catalytic hydrogenation.</p> | | | |

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**SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1,2,3-
TRIHYDROXYBENZENES USING MYO-INOSITOL-1-PHOSPHATE SYNTHASE
AND MYO-INOSITOL 2-DEHYDROGENASE**

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RELATED APPLICATIONS

The present invention is a continuation-in-part of U.S. Serial No. 5 09/274,732, filed March 23, 1999, which is hereby expressly incorporated by reference.

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SPONSORSHIP

Work on this invention was sponsored in part by the National Science Foundation Grant No. CHE963368. The Government may have certain rights in the 10 invention.

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FIELD OF THE INVENTION

The present invention is related to the production of 1,2,3,4-tetrahydroxybenzene and more specifically, to methods of producing 1,2,3,4-tetrahydroxybenzene from the bioconversion of a carbon source.

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BACKGROUND OF THE INVENTION

Polyhydroxy benzenes and quinones possessing the oxygenation pattern of 1,2,3,4-tetrahydroxybenzene 1 (Figure 1) often display biological activity. Aurantiogliocladin 2 and fumigatin 3 (Figure 1) are antibiotics. Vischer, E.B., *J. Chem. Soc.* 815 (1953); Baker, W. et al., *J. Chem. Soc.* 820 (1953); Baker, W. et al., *J. Chem. Soc.* 670 (1941). Coenzyme Q_{n+10} 4 (Figure 1) is an essential antioxidant in humans protecting low density lipoproteins from atherosclerosis-related oxidative modification. Ingold, K.U. et al., *PNAS (USA)* 90:45 (1993); Stocker, R. et al., *PNAS (USA)* 88:1646 (1991); Steinberg, D., *Circulation* 84:1420 (1991). Dillapiole 5 (Figure 1) is a pyrethrin synergist and is responsible for the 25 sedative effect of *Perilla frutescens* leaves. Honda, G. et al., *Chem. Pharm. Bull.* 36:3153 (1988); Tomar, S.S. et al., *Agric. Biol. Chem.* 50:2115 (1986).

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The current method of preparing 1,2,3,4-tetrahydroxybenzene uses pyrogallol as the synthetic starting material. Pyrogallol is converted to aminopyrogallol using a four-step synthesis. Aminopyrogallol is then hydrolyzed to 45 30 give 1,2,3,4-tetrahydroxybenzene. Conversion of pyrogallol to 1,2,3,4-tetrahydroxybenzene requires the use of such reagents as phosgene, solvents such as pyridine and xylene, and has a nitroaromatic as a synthetic intermediate.

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It would also be desirable to provide an improved method for producing derivatives of 1,2,3,4-tetrahydroxybenzene. Particularly, it would be desirable to

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provide a method for producing 1,2,3-trihydroxybenzene (pyrogallol). It would also be desirable if such a method were cost efficient and employed readily available materials. Currently, 1,2,3-trihydroxybenzene is obtained by thermal decarboxylation of gallic acid. However, gallic acid is isolated from natural sources such as gall nuts and tara powder and therefore is in limited supply.

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It would thus be desirable to provide an improved method for producing 1,2,3,4-tetrahydroxybenzene. It would also be desirable if such a method was cost-efficient, using inexpensive starting materials. It would be further desirable if the method employed non-toxic compounds and was environmentally benign.

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SUMMARY OF THE INVENTION

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A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided. In one embodiment, the bioconversion methods of the present invention comprise the steps of microbe-catalyzed conversion of a carbon source to *myo*-2-inosose followed by acid-catalyzed dehydration of *myo*-2-inosose to produce 1,2,3,4-tetrahydroxybenzene. As shown in the synthesis scheme of Figure 2, the microbe-catalyzed conversion step of the present invention requires four enzymes. In one embodiment, the microbe-catalyzed conversion comprises the conversion of a carbon source to *myo*-inositol by a recombinant microbe and the subsequent conversion of *myo*-inositol to *myo*-2-inosose catalyzed by a second microbe. In another embodiment, the recombinant microbe is *Escherichia coli* designed to cause the conversion of glucose-6-phosphate to *myo*-inositol-1-phosphate. In yet another embodiment, the conversion of *myo*-inositol to *myo*-2-inosose is catalyzed by the microbe *Gluconobacter oxydans*. Acid-catalyzed dehydration of the resulting *myo*-2-inosose yields 1,2,3,4-tetrahydroxybenzene.

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The biocatalytic synthesis of 1,2,3,4-tetrahydroxybenzene provided herein is environmentally benign, economically attractive, and utilizes abundant renewable sources as a starting material.

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Methods are also provided for the production of derivatives of 1,2,3,4-tetrahydroxybenzene, particularly Coenzyme Q 1,2,3-trihydroxybenzene (pyrogallol). In one embodiment 1,2,3-trihydroxybenzene is produced by reduction of 1,2,3,4-tetrahydroxybenzene. In a preferred embodiment, the reduction is achieved by catalytic hydrogenation followed by hydrolysis.

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Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

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BRIEF DESCRIPTION OF THE DRAWINGS

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The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

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Figure 1 is an illustration showing the structures of products that can be derived from 1,2,3,4-tetrahydroxybenzene;

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Figure 2 is a schematic illustrating the bioengineered synthesis scheme of the present invention for producing 1,2,3,4-tetrahydroxybenzene;

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Figure 3 is a graph showing the production of *myo*-inositol in comparison to cell dry weight;

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Figure 4 is a schematic illustrating the conventional synthetic scheme for synthesizing 1,2,3,4-tetrahydroxybenzene; and

Figure 5 is a schematic illustrating the synthesis scheme for converting 1,2,3,4-tetrahydroxybenzene to Coenzyme Q.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

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A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided herein. Methods of producing 1,2,3,4-tetrahydroxybenzene from a carbon source based on the synthesis scheme are also provided. In one embodiment, a method is provided wherein the carbon source is converted to *myo*-inositol by a recombinant microbe, the *myo*-inositol is further converted to *myo*-2-inosose by a second microbe, followed by acid-catalyzed dehydration of *myo*-2-inosose to produce 1,2,3,4-tetrahydroxybenzene.

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Novel methods are also provided for the production of derivatives of 1,2,3,4-tetrahydroxybenzene, particularly 1,2,3-trihydroxybenzene (pyrogallol). In one embodiment 1,2,3-trihydroxybenzene is produced by reduction of 1,2,3,4-tetrahydroxybenzene. In a preferred embodiment, the reduction is achieved by catalytic hydrogenation of 1,2,3,4-tetrahydroxybenzene followed by acid catalyzed hydrolysis to yield 1,2,3-trihydroxybenzene. In a more preferred embodiment, the hydrogenation is catalyzed by Rh/Al₂O₃. These methods take advantage of inexpensive and abundant carbon sources as starting materials to produce 1,2,3,4-tetrahydroxybenzene which can then be converted to 1,2,3-trihydroxybenzene.

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Although microbe-catalyzed conversion of a carbon source to *myo*-inositol by a recombinant microbe followed by conversion of the *myo*-inositol to *myo*-2-inosose by a second microbe is described in detail herein, in an alternative embodiment, a single recombinant microbe is employed to convert a carbon source directly to *myo*-2-inosose which is then converted to 1,2,3,4-tetrahydroxybenzene by an acid-catalyzed dehydration. This single-microbe conversion may be carried out by any type of microbe sufficiently engineered to produce the desired outcome.

In another alternative embodiment, a recombinant microbe catalyzes the conversion of a carbon source to D-2,3-diketo-4-deoxy-*epi*-inositol which is subsequently converted to 1,2,3,4-tetrahydroxybenzene by an acid-catalyzed dehydration. D-2,3-Diketo-4-deoxy-*epi*-inositol is an intermediate in the microbial catabolism of *myo*-inositol as well as a likely intermediate in the acid-catalyzed conversion of *myo*-2-inosose into 1,2,3,4-tetrahydroxybenzene.

The bioconversion methods of the present invention are carried out under conditions of time, temperature, pH, nutrient type and concentration, aeration conditions and glucose concentrations, to provide maximal conversion of the carbon source to 1,2,3,4-tetrahydroxybenzene. As described in detail in Specific Example 1, in a preferred embodiment, a fed-batch fermentor is used to convert the carbon source to *myo*-inositol, followed by isolation of *myo*-inositol, e.g., deionization and decolorization of the fermentation broth and precipitation by the addition of an organic solvent. The isolated *myo*-inositol is then converted to *myo*-inosose followed by isolation of the *myo*-2-inosose, e.g., precipitation of the *myo*-2-inosose from the culture broth. The fed-batch fermentor process and the precipitation methods are also known to those skilled in the art.

As used herein, the phrase "carbon source" is meant to include biomass-derived carbon sources including, but not limited to, xylose, arabinose, glycerol, glucose and the intermediates (e.g., dicarboxylic acids) in the Krebs cycle, either alone or in combination. In a preferred embodiment, the carbon source is glucose.

In one embodiment, a recombinant *E. coli* microbe is employed in the methods of the present invention. In a preferred embodiment, the *E. coli* comprises a non-functional *serA* locus. This recombinant *E. coli*, designated, JWF1, may further comprise a plasmid carrying an *INO1* gene insert and a *serA* gene insert. The *INO1* gene encodes *myo*-inositol-1-phosphate synthase which converts glucose-6-phosphate to *myo*-inositol-1-phosphate. In a preferred embodiment, the *INO1* gene is from *Saccharomyces cerevisiae*. Overexpression of *myo*-inositol-1-

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phosphate synthase will increase carbon flow into the *myo*-inositol pathway. This recombinant microbe is capable of converting glucose to *myo*-inositol.

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In another embodiment, the *myo*-inositol produced by the first recombinant microbe, is converted to *myo*-2-inosose by a second microbe. This second microbe 5 can either be a recombinant microbe or a naturally occurring microbe. A recombinant microbe comprises a plasmid carrying the *io/G* gene insert. The *io/G* gene insert encodes the enzyme inositol dehydrogenase, which catalyzes the conversion of *myo*-inositol to *myo*-2-inosose. Alternatively, the *io/G* gene insert is inserted directly into the genome of the recombinant microbe. Preferably, the *io/G* 10 gene is isolated from *Bacillus subtilis*. In a preferred embodiment, the second microbe is a naturally occurring microbe that express inositol dehydrogenase activity. Examples of such microbes include, but are not limited to, *Bacillus subtilis* and *Gluconobacter oxydans*. In a preferred embodiment, the second microbe is *G. oxydans*, which converts *myo*-inositol to *myo*-2-inosose without loss of the *myo*-2- 15 inosose product to further catabolism.

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In a preferred embodiment, the recombinant *E. coli* comprises plasmid pAD1.88A carrying an *INO1* gene insert and a *serA* gene insert. As described above, the *INO1* gene insert encodes *myo*-inositol-1-phosphate synthase which converts glucose-6-phosphate to *myo*-inositol-1-phosphate, thus increasing the 20 carbon flow into the *myo*-inositol pathway. Due to a mutation in the *E. coli* genomic *serA* locus required for L-serine biosynthesis, growth in minimal salts medium and plasmid maintenance follows from expression of plasmid-localized *serA*. The *serA* insert thus allows microbial growth in minimal salts medium, 25 distinguishing the microbes containing the plasmid from non-plasmid containing microbes.

In an alternative embodiment, the recombinant *E. coli* comprises a plasmid carrying an *INO1* gene insert, an *io/G* gene insert and a *serA* gene insert. As described above, the *io/G* gene insert encodes inositol dehydrogenase which catalyzes the conversion of *myo*-inositol to *myo*-2-inosose. In a preferred 30 embodiment, the plasmid also carries the gene insert for inositol monophosphatase. While not wishing to be bound by theory, hydrolysis of *myo*-inositol-1-phosphate to produce *myo*-inositol can occur in the cytosol or periplasm. If a cytoplasmic phosphatase hydrolyzes *myo*-inositol-1-phosphate, plasmid-localized *INO1* and *io/G* 35 will lead to *myo*-2-inosose synthesis. Periplasmic phosphatase activity would result in periplasmic production of *myo*-inositol while inositol dehydrogenase expression

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is localized in the cytoplasm. Transport of *myo*-inositol from the periplasm into the cytoplasm is unlikely in *E. coli* given that this microbe does not catabolize *myo*-inositol. To correct for periplasmic phosphatase activity, plasmid-localization of genes encoding mammalian inositol monophosphatase, which have been cloned,

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sequenced, and successfully expressed in *E. coli*, would be desirable. Diehl, R.E. et al., *J. Biol. Chem.* 265:5946 (1990); McAllister, G. et al., *Biochem. J.* 284:749 (1992). Because of the specificity of this enzyme for *myo*-inositol-1-phosphate, a molecule which is not a normal metabolite in *E. coli*, cytoplasmic expression of the cDNA encoding inositol monophosphatase in *E. coli* should not be problematic. An 10 *E. coli* comprising a plasmid carrying both the *INO1* gene insert and the *io/G* gene insert and the gene for inositol monophosphatase can convert glucose directly to *myo*-2-inosose. The *myo*-2-inosose can then be converted to 1,2,3,4-tetrahydroxybenzene by an acid-catalyzed dehydration.

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In another embodiment, the recombinant *E. coli*, designated JWF1, 15 comprises a plasmid carrying an *INO1* gene insert, an *io/G* gene insert, and a *serA* gene insert. This recombinant microbe is capable of converting glucose to *myo*-2-inosose. In a preferred embodiment, the recombinant *E. coli* comprises plasmid pAD2.28A carrying an *INO1* gene insert, an *io/G* gene insert and a *serA* gene insert.

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Examples of these recombinant microbes, *E. coli* JWF1/pAD1.88A and 20 JWF1/pAD2.28A, are described in Specific Examples 1 and 4, respectively, and have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20582, under the terms of the Budapest Treaty, and accorded the ATCC designation numbers 207153 and 207154, respectively. The deposit will be maintained in the ATCC depository, which is a 25 public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of a patent, whichever is longer, and will be replaced if the deposit becomes depleted or non-viable during that period. Samples of the deposit will become available to the public and all restrictions imposed on access to the deposit will be removed upon grant of a patent on this application.

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40 In yet another embodiment, a recombinant *E. coli* is employed to convert glucose to D-2,3-diketo-4-deoxy-*epi*-inositol. Such a recombinant *E. coli* comprises a plasmid carrying the *INO1* gene insert, the *io/G* gene insert and the gene insert encoding for the enzyme, *myo*-2-inosose dehydratase. The plasmid may further 45 comprise the gene insert for inositol monophosphatase. *Myo*-2-inosose dehydratase 50 catalyzes the conversion of *myo*-2-inosose to D-2,3-diketo-4-deoxy-*epi*-inositol 55

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which can undergo an acid-catalyzed dehydration to yield 1,2,3,4-tetrahydroxybenzene. While not wishing to be bound by theory, D-2,3-diketo-4-deoxy-*epi*-inositol is likely the first intermediate in the acid-catalyzed conversion of *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene. Acid-catalyzed aromatization of D-2,3-diketo-4-deoxy-*epi*-inositol would eliminate a step and may lead to higher yields of 1,2,3,4-tetrahydroxybenzene as compared to the acid-catalyzed dehydration of *myo*-2-inosose.

It will be appreciated that the *INO1* gene, *ioG* gene, *serA* gene and the genes encoding inositol dehydrogenase, inositol monophosphatase and/or *myo*-2-inosose dehydratase, can be inserted directly into the *E. coli* genome. Such a recombinant *E. coli* would not require a plasmid to produce significant amounts of *myo*-inositol, *myo*-2-inosose or D-2,3-diketo-4-deoxy-*epi*-inositol.

Although *E. coli* is specifically described herein as the microbe for carrying out the methods of the present invention, it will be appreciated that any microorganism such as the common types cited in the literature and known to those skilled in the art, may be employed, provided the microorganism can be altered to effect the desired conversion (e.g., carbon source to *myo*-inositol, carbon source to *myo*-2-inosose, carbon source to D-2,3-diketo-4-deoxy-*epi*-inositol, *myo*-inositol to *myo*-2-inosose, etc.). Thus it is envisaged that many types of fungi, bacteria and yeasts will work in the methods of the present invention. Such microorganisms may be developed, for example, through selection, mutation, and/or genetic transformation processes with the characteristic and necessary capability of converting one constituent of the synthesis scheme of the present invention to another. Methods for such development are well known to the skilled practitioner.

In order to carry out the bioconversion methods of the present invention, a solution containing a carbon source is contacted with the recombinant or wild-type microbe to form a bioconversion mixture which is maintained under appropriate conditions to promote the conversion of the carbon source to the desired constituent, e.g., *myo*-inositol or *myo*-2-inosose. In a preferred embodiment, the bioconversion mixture is maintained at a temperature of about 30°C to about 37°C and a pH of about 6.5 to about 7.5. It is preferred that the bioconversion mixture also contain other substances necessary to promote viability of the microbes such as mineral salts, buffers, cofactors, nutrient substances and the like. The more general requirements for the maintenance of viability of microorganisms are well known and specific requirements for maintaining the viability of specific organisms

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are also well known as documented in the literature, or are otherwise easily determined by those skilled in the art.

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In another embodiment, the *myo*-inositol produced in the fermentation broth of the first bioconversion with the first microbe can be isolated before being utilized 5 in the second bioconversion. The isolation can be a total isolation to provide pure *myo*-inositol as described in Specific Example 1. The isolation can also be a partial isolation where the fermentation broth is deproteinized and decolorized before use 10 in the second bioconversion. Such deproteinization and decolorization are well known to those skilled in the art. Further purification of the *myo*-inositol can be obtained by concentration of the deproteinized/decolorized fermentation broth to a volume where the *myo*-inositol concentration is greater than 50 g/L. At 15 concentrations greater than 50 g/L, the *myo*-inositol can be precipitated out by the addition of methanol. By way of non-limiting example, *myo*-inositol can be isolated from the fermentation broth by the following four steps: ultrafiltration to remove 20 cells and proteins; decolorization by activated charcoal; concentration of the fermentation broth to give a *myo*-inositol concentration greater than 50 g/L; and precipitation of *myo*-inositol by the addition of methanol. The *myo*-inositol precipitate can be washed, resuspended in buffer or water, and used in the second 25 bioconversion to *myo*-2-inosose.

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20 Novel methods for converting 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene are also provided. In one embodiment, a method is provided 25 wherein 1,2,3,4-tetrahydroxybenzene is reduced to 1,2,3-trihydroxybenzene.

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35 In one embodiment, the 1,2,3,4-tetrahydroxybenzene is converted to 1,2,3-trihydroxybenzene by hydrogenation in the presence of a catalyst followed by acid 40 catalyzed hydrolysis. In a preferred embodiment, the catalyst is Rh/Al₂O₃. The amount of catalyst required and the conditions required for hydrogenation (e.g., pressure, time) are well known to the skilled practitioner.

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45 In another embodiment, the hydrogenation reaction is carried out in an aqueous 1,2,3,4-tetrahydroxybenzene solution. In a preferred embodiment, the 50 aqueous 1,2,3,4-tetrahydroxybenzene solution will be free of any compounds 55 known to quench the hydrogenolysis catalyst. In a more preferred embodiment, the aqueous 1,2,3,4-tetrahydroxybenzene solution is comprised of isolated 1,2,3,4-tetrahydroxybenzene and water.

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50 In order to more fully demonstrate the advantages arising from the present 55 invention, the following examples are set forth. It is to be understood that the

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following is by way of example only and is not intended as a limitation on the scope
of the invention.

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SPECIFIC EXAMPLE 1

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Synthesis Of 1,2,3,4-Tetrahydroxybenzene From Glucose.

5 I. Results

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A synthetic route (Figure 2) has now been elaborated which provides
convenient access to 1,2,3,4-tetrahydroxybenzene via *myo*-inositol intermediacy.
The general utility of this route is demonstrated by a concise synthesis of coenzyme
Q₁₀, 4. While the shikimate pathway and polyketide biosynthesis have traditionally
10 provided biocatalytic access to aromatic chemicals, syntheses of 1,2,3,4-
20 tetrahydroxybenzene 1 and coenzyme Q₁₀, 4 are distinguished by the recruitment
of *myo*-inositol biosynthesis.

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Synthesis of *myo*-inositol by *E. coli* JWF1/pAD1.88A begins with D-glucose
25 uptake and conversion to D-glucose-6-phosphate catalyzed by the *E. coli*
phosphotransferase system (Postma, P.W. et al., In *Escherichia coli and Salmonella*,
2nd ed., Neidhardt, F.C. et al., Eds., ASM: Washington, Vol. 1, p. 1149 (1996))
where phosphoenolpyruvate is the source of the transferred phosphoryl group. D-
30 Glucose-6-phosphate then undergoes cyclization to *myo*-inositol 1-phosphate
catalyzed by *myo*-inositol 1-phosphate synthase. This enzyme activity, which
20 results from expression of the *Saccharomyces cerevisiae* *INO1* gene (Dean-Johnson,
M. et al., *J. Biol. Chem.* 264:1274 (1989)) on plasmid pAD1.88A, varied
significantly (0.022, 0.043, 0.018, and 0.009 μmol/min/mg at 18 h, 30 h, 42 h,
35 and .54 h, respectively) over the course of the fermentation.

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E. coli JWF1/pAD1.88A synthesized 21 g/L *myo*-inositol (solid bars, Figure
25 3) and 4 g/L *myo*-inositol-1-phosphate (open bars, Figure 3) in 11% combined yield
(mol/mol) from D-glucose under fed-batch fermentor conditions. Both *myo*-inositol
and *myo*-inositol-1-phosphate accumulated in the culture supernatant. In
eucaryotes, hydrolysis of *myo*-inositol-1-phosphate to *myo*-inositol is catalyzed by
the enzyme inositol monophosphatase. McAllister, G. et al., *Biochem. J.* 284:749
30 (1992). Phosphoester hydrolysis was fortuitously catalyzed in *E. coli*
JWF1/pAD1.88A by unidentified cytosolic or periplasmic phosphatase activity.

Oxidation of *myo*-inositol to *myo*-2-inosose, the next step in the conversion
of D-glucose into 1,2,3,4-tetrahydroxybenzene 1, is the first catabolic step when
myo-inositol is used as a sole source of carbon for growth and metabolism by
35 microbes such as *Bacillus subtilis*. Yoshida, K.-I. et al., *J. Bacteriol.* 179:4591

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(1997). *myo*-Inositol can also be oxidized by *Gluconobacter oxydans* without loss of product *myo*-2-inosose to catabolism. Posternak, T., *Bioch. Prep.* 2:57 (1952). Accordingly, incubation of *G. oxydans* ATCC 621 in medium containing microbe-synthesized *myo*-inositol led to the formation of *myo*-2-inosose (Scheme 1) in 95% isolated yield.

Inososes have been thought to be stable under acidic conditions and reactive under basic conditions with reported aromatizations resulting from successive β -eliminations being dominated by formation of 1,2,3,5-tetrahydroxybenzene. Posternak, T., *The Cyclitols*, Holden-Day: San Francisco, Chap. 8 (1965); Angyal, S.J. et al., *Carbohydr. Res.* 76:121 (1979). However, it was observed that *myo*-2-inosose was reactive under acidic conditions with no apparent formation of 1,2,3,5-tetrahydroxybenzene. Refluxing *G. oxydans*-produced *myo*-2-inosose for 9 h in degassed, aqueous 0.5 M H₂SO₄ under argon cleanly afforded 1,2,3,4-tetrahydroxybenzene in 66% isolated yield.

15 II. Materials And Methods

General. ¹H NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts for ¹H NMR spectra are reported (in parts per million) relative to internal tetramethylsilane (Me₄Si, δ = 0.0 ppm) with CDCl₃ as solvent, to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (TSP, δ = 0.0 ppm) when D₂O was the solvent, and to acetone (CHD₂COCD₃, δ = 2.04 ppm) with d₆-acetone. ¹³C NMR spectra were recorded at 75 MHz. Chemical shifts for ¹³C NMR spectra are reported (in parts per million) relative to CDCl₃ (δ = 77.0 ppm), relative to CD₃COCD₃ (δ = 29.8 ppm), and relative to internal CH₃OH (δ = 49.0 ppm) or internal CH₃CN (δ = 1.4 ppm) in D₂O. FAB mass spectra were performed by University of South Carolina (Columbia, SC). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

Radial chromatography was carried out with a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60 PF₂₅₄ containing gypsum (E. Merck). Silica gel 60 (40 - 63 μ m E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, E. Merck or Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) followed by heating. Dimethylformamide, N-methylformanilide and acetone were dried and stored over activated Linde 4 \AA

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molecular sieves under Ar. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. *n*-Hexanes and TMEDA were distilled from sodium under Ar and stored over activated Linde 4Å molecular sieves under Ar. Organic solutions of products were dried over MgSO₄.

For ¹H NMR quantitation of solute concentrations during microbial synthesis of *myo*-inositol and *myo*-2-inosose, solutions were concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D₂O, and then redissolved in D₂O containing a known concentration of TSP purchased from Lancaster Synthesis Inc. Concentrations were determined by comparison of integrals corresponding to each compound with the integral corresponding to TSP ($\delta = 0.00$ ppm) in the ¹H NMR. Protein concentrations were determined using the Bradford dye-binding procedure (Bradford, M.M., *Anal. Biochem.* 72:248 (1979)) by comparison with a standard curve prepared with bovine serum albumin. Protein assay solution was purchased from Bio-Rad. *E. coli* DH5 α is available from Gibco BRL.

Culture Medium. All culture solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NaCl (0.5 g) and NH₄Cl (1 g). M9 minimal medium (1 L) consisted of 1 L of M9 salts containing D-glucose (10 g), MgSO₄ (0.12 g), and thiamine hydrochloride (0.001 g). Ampicillin was added (0.05 g/L) where indicated. Solutions of M9 salts, MgSO₄, and glucose were autoclaved individually and then mixed. Ampicillin was sterilized through a 0.22 μ m membrane. Solid medium was prepared by addition of 1.5% (w/v) Difco agar to medium.

Fermentation medium (1 L) contained K₂HPO₄ (7.5 g), ammonium iron(III) citrate (0.3 g), citric acid monohydrate (2.1 g), and concentrated H₂SO₄ (1.2 mL). The fermentation medium was adjusted to pH 7.0 by addition of concentrated NH₄OH before autoclaving. The following supplements were added immediately prior to initiation of the fermentation: D-glucose (20 g), MgSO₄ (0.24 g), and trace minerals including (NH₄)₆(Mo₇O₂₄)·4H₂O (0.0037 g), ZnSO₄·7H₂O (0.0029 g), H₃BO₃ (0.0247 g), CuSO₄·5H₂O (0.0025 g), and MnCl₂·4H₂O (0.0158 g). D-Glucose and MgSO₄ were autoclaved separately while trace minerals were sterilized through 0.22 μ m membranes prior to addition to the medium.

Genetic Manipulations. Standard procedures were used for the construction, purification, and analysis of plasmid DNA. Sambrook, J. et al., *Molecular Cloning*

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A Laboratory Manual, Cold Spring Harbor Laboratory Press: New York (1989). *E. coli* DH5 α served as the host strain for all plasmid constructions. The *INO1* open reading frame was amplified from pJH318 (Hirsch, J.P. et al., *Mol. Cell. Biol.* 6:3320 (1986)) using PCR. Inclusion of *Eco*RI recognition sequences facilitated localization of *INO1* into the *Eco*RI site in pJF118EH (Fürste, J.P. et al., *Gene* 48:119 (1986)) to afford pAD1.45A. Transcription of *INO1* in pAD1.45A utilized the vector-encoded *tac* promoter (P_{lac}) which was regulated by vector-encoded expression of *lacZ*. Digestion of pD2625 with *Dra*I and *Eco*RV liberated a 1.9 kb *serA* fragment that was subsequently ligated into the *Sma*I site of pAD1.45A to provide pAD1.88A.

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Localization of the 1.9 kb *serA* fragment obtained from pD2625 into pMAK705 (Hamilton, C.M. et al., *J. Bacteriol.* 171:4617 (1989)) provided pLZ1.68A. Linearization of pLZ1.68A at the unique *Bam*HI site internal to *serA* followed by treatment with Klenow fragment and dNTP's and recombination afforded pLZ1.71A. Homologous recombination (Ohta, K. et al., *Appl. Environ. Microbiol.* 57:893 (1991)) of the resulting non-functional *serA* locus of pLZ1.71A into RB791 afforded JWF1.

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E. coli JWF1 was prepared by homologous recombination of a non-functional *serA* gene into *E. coli* RB791(W3110 *lacZ*). Localization of the 1.9 kb *serA* fragment obtained from pD2625 into pMAK705 (Hamilton, C.M. et al., *J. Bacteriol.* 171:4617 (1989)) provided pLZ1.68A. Linearization of pLZ1.68A at the unique *Bam*HI site internal to *serA* followed by treatment with Klenow fragment and dNTP's and recombination afforded pLZ1.71A. Homologous recombination (Ohta, K. et al., *Appl. Environ. Microbiol.* 57:893 (1991)) of the resulting non-functional *serA* locus of pLZ1.71A into RB791 afforded JWF1.

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myo-Inositol-1-phosphate synthase activity. Partial purification of cellular lysate was required to quantify myo-inositol-1-phosphate synthase activity over background cellular phosphatase activity. Cells were collected from 30 mL of fermentation broth by centrifugation at 2000g for 6 min at 4 °C. Cells were resuspended in 10 mL of resuspension buffer consisting of Tris-HCl (20 mM), pH 7.4, NH₄Cl (10 mM), 2-mercaptoethanol (β -ME, 10 mM), phenylmethylsulphonyl fluoride (PMSF, 2 mM), and EDTA (1 mM). Resuspended cells were frozen at -80 °C for up to 4 days until purification was carried out.

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Thawed cells were lysed by two passages through a French press at 2000 psi. Cellular debris was removed by centrifugation at 30000g for 30 min at 4 °C. Clarified cellular lysate containing approximately 200 mg of protein was loaded onto a DEAE cellulose column (5 x 25 cm) at 4 °C. The column was eluted with a step gradient of NH₄Cl in the following buffer (Buffer A): Tris-HCl (20 mM), pH 7.4, β -ME (10 mM), PMSF (1 mM), and EDTA (1 mM). The step gradient consisted of 20 mL of Buffer A with NH₄Cl (10 mM), 45 mL of Buffer A with NH₄Cl (90 mM), and 100 mL of Buffer A with NH₄Cl (150 mM). Fractions (9 mL) were collected throughout the step gradient. Fractions 10-18 were collected and concentrated to

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less than 5 mL using an Amicon Ultrafiltration Stirred Cell equipped with a PM10 membrane. Concentrated protein (1.5-2.0 mg) was used to measure *myo*-inositol-1-phosphate synthase activity. The *myo*-inositol-1-phosphate synthase activity was measured as previously reported (Migaud, M.E. et al., *J. Am. Chem. Soc.* 118:495 (1996)) except that the assay solution contained Tris/HCl (20 mM), pH 7.4, NH₄Cl (2 mM), and DTT (0.2 mM).

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myo-Inositol. Fermentations employed a 2.0 L working capacity B. Braun MD2 culture vessel. Utilities were supplied by a B. Braun Biostat MD controlled by a Dell Optiplex Gs+ 5166 personal computer, equipped with B. Braun MFCS/Win software. Temperature, pH, and glucose feeding were controlled with PID control loops. Temperature was maintained at 33 °C. pH was maintained at 7.0 by addition of concentrated NH₄OH or 2 N H₂SO₄. Dissolved oxygen (D.O.) was measured using a Mettler-Toledo 12 mm sterilizable O₂ sensor fitted with an Ingold A-type O₂ permeable membrane. D.O. was maintained at 10% air saturation. Antifoam (Sigma 204) was added manually as needed.

Inoculants were started by introduction of a single colony of JWFI/pAD1.88A into 100 mL M9 medium containing ampicillin. The culture was grown at 37 °C with agitation at 250 rpm for 15 h and then transferred to the fermentation vessel. The initial glucose concentration in the fermentation medium was 20 g/L. Three staged methods were used to maintain D.O. levels at 10% air saturation during each fermentor run. With the airflow at an initial setting of 0.06 L/L/min, D.O. concentration was maintained by increasing impeller speed from its initial set point of 50 rpm to its preset maximum of 940 rpm. Approximately 7 h was required for the impeller speed to increase to 940 rpm. With the impeller constant at 940 rpm, the mass flow controller then maintained D.O. levels by increasing the airflow rate from 0.06 L/L/min to its preset maximum of 1.0 L/L/min over approximately 1.5 h. At constant impeller speed and constant airflow rate, D.O. levels were maintained at 10% saturation for the remainder of the fermentation by oxygen sensor-controlled glucose feeding. At the beginning of this stage, D.O. levels fell below 10% air saturation due to residual initial glucose in the medium. This lasted for approximately 50 min before glucose (60% w/v) feeding started. The PID control parameters were set to 0.0 (off) for the derivative control (r_D) and 999.9 s (minimum control action) for integral control (r_I). X_s was set to 950% to achieve a K_c of 0.1.

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Samples (6 mL) of fermentation broth were taken at 6 h intervals starting at 12 h. Isopropyl- β -D-thiogalactopyranoside (4.8 mg) was added when both the impeller speed and airflow had reached the maximum settings, and again at 12 h and every 6 h thereafter. Cell densities were determined by dilution of fermentation

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broth with water (1:100) followed by measurement of absorption at 600 nm (OD_{600}). Dry cell weight (g/L) was obtained using a conversion coefficient of 0.43 g/L/ OD_{600} . Fermentation broth was centrifuged to remove cells. Solute concentrations in cell-free broth were determined by 1H NMR. Fermentation broth (30 mL) was removed at designated times for assay of *myo*-inositol-1-phosphate

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synthase activity. The final concentration of *myo*-inositol at 54 h was 20.9 g/L synthesized in 8.7% yield (mol/mol) from glucose.

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The fermentation broth (950-1200 mL) was centrifuged at 18000g for 35 min at 4 °C and the cells discarded. The resulting supernatant was acidified to pH 2.0 with concentrated H_2SO_4 and then centrifuged at 18000g for 20 min to remove precipitated proteins. The clear yellow supernatant was neutralized with concentrated NH_4OH . The solution was decolorized with Darco KB-B activated carbon (10 g/L) for 4 h with agitation at 50 rpm and subsequently filtered through Whatman 2 filter paper. The filtered material was washed with an additional 200 mL of water.

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The combined filtrates were applied to a column of AG1-x8 (acetate form, 5 cm x 20 cm) at 4 °C and eluted with 1 L H_2O . The entire eluent (approximately 2.3 L) was then run through a column of Dowex 50 (H⁺ form, 5 cm x 20 cm) at 4 °C and eluted with 500 mL H_2O . The resulting solution (approximately 2.8 L) was concentrated to 200 mL by boiling and then concentrated to dryness under reduced pressure. The resulting powder was dissolved in a minimal volume of H_2O , diluted with 6 volumes of MeOH, and stored at 4 °C to crystallize. Crystals were collected after a few days, washed with MeOH, allowed to air dry overnight, and dried under vacuum to yield white crystals (78 % recovery based on inositol quantified in crude fermentation broth). 1H NMR (D_2O) δ 4.06 (dd, $J=3, 3$ Hz, 1 H), 3.61 (dd, $J=10,$ 9 Hz, 2 H), 3.53 (ddd, $J=10, 3, 1$ Hz, 2 H), 3.28 (ddd, $J=9, 9, 1$ Hz, 1 H). ^{13}C NMR (D_2O) δ 45.3, 43.4, 43.2, 42.1.

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myo-2-Inosose. Angyal, S.J. et al. *Carbohydr. Res.* 76:121 (1979); Posternak, T., *Biochem. Prep.* 2:57 (1952). A solution containing sorbitol (1.0 g) and yeast extract (0.05 g) in 10 mL distilled, deionized water was autoclaved for 35 25 min and cooled to room temperature. After inoculation with *Gluconobacter*

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oxydans ATCC 621 the culture was incubated in an orbital shaker at 200 rpm for 24 h at 30 °C. This *G. oxydans* culture was subsequently added to a second sterile solution containing *myo*-inositol (12.0 g, 66.7 mmol), D-sorbitol (0.4 g), and yeast extract (2.0 g) in 400 mL distilled, deionized water. After incubation in an orbital shaker at 200 rpm for 48 h at 30 °C, cells were removed by centrifugation. The resulting culture supernatant was concentrated to 75 mL, MeOH (400 mL) added, and the solution maintained at -20 °C for 12 h. Precipitate which formed was filtered, washed with MeOH, and dried to afford *myo*-2-inosose as a white powder (8.17 g, 69%). A second crop of *myo*-2-inosose (3.09 g, 26%) was obtained after maintaining the filtrate at -20 °C for an additional 12 h. mp 188-192

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°C. ¹H NMR (D₂O): δ 4.25 (d, J = 10 Hz, 2 H), 3.66 (dd, J = 9, 9 Hz), 3.26 (m, 2 H). ¹³C NMR (D₂O): δ 206.0, 94.3, 76.2, 74.5, 74.1, 74.0, 73.3, 73.2.

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1,2,3,4-Tetrahydroxybenzene 1. A solution of *myo*-2-inosose (11.0 g, 61.2 mmol) in 310 mL of degassed 0.5 M H₂SO₄ was refluxed under Ar. After 9 h, the solution was cooled to 4 °C and then adjusted to pH 4 by addition of saturated aqueous NaHCO₃. Concentration of the reaction solution to 100 mL was followed by continuous liquid-liquid extraction for 18 h using *t*-butyl methyl ether (500 mL). Upon concentration of the organic layer to 100 mL, a precipitate formed which was filtered, washed with cold hexanes, and dried to afford 1 (4.72 g, 54%) as a tan powder. Addition of hexanes (300 mL) to the filtrate followed by filtering, washing, and drying of the resulting precipitate afforded additional 1 (1.08 g, 12%). mp 162 - 164 °C. ¹H NMR (*d*₆-acetone): δ 7.24 (s, 4 H), 6.20 (s, 2 H). ¹³C NMR (*d*₆-acetone): δ 139.7, 134.7, 106.2. Anal. Calcd for C₆H₆O₄: C, 50.71; H, 4.23. Found: C, 50.63; H, 4.32. HRMS (FAB) calcd for C₆H₆O₄ (M + H⁺): 142.0266. Found: 142.0268.

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SPECIFIC EXAMPLE 2 Chemical Synthesis Of 1,2,3,4-Tetrahydroxybenzene

I. Results

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Conversion of D-glucose into 1,2,3,4-tetrahydroxybenzene 1 is a three step synthesis. 1,2,3,4-Tetrahydroxybenzene 1 has historically been obtained from pyrogallol 6 by a longer route (Figure 4) involving synthesis and subsequent hydrolysis of aminopyrogallol 7. Leston, G., In *Kirk-Othmer Encyclopedia of Chemical Technology*: Fourth Ed., Kroschwitz, J.I. et al. Ed., Wiley: New York, Vol. 19, p. 778 (1996); Einhorn, A. et al., *Ber.* 37:110 (1904). Due to the tedious nature of this synthesis (Einhorn, A. et al., *Ber.* 37:110 (1904)), two alternate

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routes (Figure 4) were developed to obtain authentic samples of 1,2,3,4-tetrahydroxybenzene 1. Low-yielding, direct hydroxylation of protected pyrogallol 8 or higher-yielding, indirect oxidation via formyl 10 intermediacy yielded, respectively, quinone 9 and phenol 11. Hydrogenation of 9 and 11 afforded products which were identical to 1,2,3,4-tetrahydroxybenzene 1 synthesized (Figure 2) from D-glucose.

II. Materials And Methods

General. ^1H NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts for ^1H NMR spectra are reported (in parts per million) relative to internal tetramethylsilane (Me_3Si , $\delta = 0.0$ ppm) with CDCl_3 as solvent, to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (TSP, $\delta = 0.0$ ppm) when D_2O was the solvent, and to acetone (CH_3COCD_3 , $\delta = 2.04$ ppm) with d_6 -acetone. ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts for ^{13}C NMR spectra are reported (in parts per million) relative to CDCl_3 ($\delta = 77.0$ ppm), relative to CD_3COCD_3 ($\delta = 29.8$ ppm), and relative to internal CH_3OH ($\delta = 49.0$ ppm) or internal CH_3CN ($\delta = 1.4$ ppm) in D_2O . FAB mass spectra were performed by University of South Carolina (Columbia, SC). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

Radial chromatography was carried out with a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60 PF₂₅₄ containing gypsum (E. Merck). Silica gel 60 (40 - 63 μm E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, E. Merck or Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) followed by heating. Dimethylformamide, N-methylformanilide and acetone were dried and stored over activated Linde 4 Å molecular sieves under Ar. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. n-Hexanes and TMEDA were distilled from sodium under Ar and stored over activated Linde 4 Å molecular sieves under Ar. Organic solutions of products were dried over MgSO_4 .

Hydrogenation of 2,3-dibenzylxy-1,4-benzoquinone 9. A solution of 9 (0.18 g, 0.56 mmol) in EtOH (7.0 mL) was stirred with 10 % Pd on C (0.050 g) at room temperature under H_2 (1.0 atm) for 3 h. The solution was filtered through Celite ^{*} and concentrated to afford a tan solid (0.079 g, 99%) which was identical

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by ^1H and ^{13}C NMR to 1 obtained from *myo*-2-inosose. Hydrogenation of 2,3,4-tribenzyloxyphenol 11. A solution of 11 (5.8 g, 14.1 mmol) in EtOH (100 mL) was stirred with 10% Pd on C (1.0 g) at room temperature under H_2 (1.0 atm) for 2 h. The solution was filtered through Celite[®] and concentrated. The residue was purified by flash chromatography (MeOH/CH₂Cl₂, 1:9, v/v) affording a product which was identical by ^1H and ^{13}C NMR to 1 obtained from *myo*-2-inosose.

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1,2,3-Tribenzyloxybenzene 8. Lowe, W. et al., *Arch. Pharm. (Weinheim)* 327:255 (1994). Benzyl bromide (57 mL, 0.481 mol) and then K₂CO₃ (100 g, 0.725 mol) were added to a solution of pyrogallol (20 g, 0.159 mol) in 200 mL dry,

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degassed acetone under Ar. This reaction mixture was subsequently stirred for 30 min at room temperature and then for 24 h at reflux under Ar. A solution of NaOH (1.6 g) in MeOH (32 mL) was then added and the reaction refluxed for an additional 30 min. After cooling to room temperature, solids were filtered and washed with acetone. The filtrate was concentrated and the residue recrystallized from MeOH to afford 8 (52 g, 83%) as an off-white solid. mp 67 - 68 °C. ^1H NMR (CDCl₃): δ 7.44-7.19 (m, 15 H), 6.85 (dd, J = 9, 9 Hz, 1 H), 6.57 (d, J = 8.2 Hz, 2 H), 5.05 (s, 2 H), 5.02 (s, 4 H). ^{13}C NMR (CDCl₃): δ 152.9, 138.4, 137.8, 137.0, 128.4, 128.3, 128.0, 127.8, 127.6 (2), 127.3, 127.2, 123.5, 107.7, 75.0, 70.9.

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2,3-Dibenzyloxy-1,4-benzoquinone 9. To a solution of 1,2,3-tribenzyloxybenzene 8 (2.0 g, 5.0 mmol) in HOAc (30 mL), K₃Fe(CN)₆ (0.82 g, 2.5 mmol) and 30% H₂O₂ (1.3 g, 11.5 mmol) were added and the resulting solution stirred at room temperature for 18 h. The solution was diluted with 50 mL CH₂Cl₂ and the organic layer subsequently washed with H₂O, saturated aqueous NaHCO₃ and brine. Drying and concentration resulted in a red oil. Purification by radial chromatography (2 mm thickness, EtOAc/hexane, 1:19, v/v) afforded 9 as a red oil. ^1H NMR (CDCl₃): δ 7.36-7.32 (m, 10 H), 6.58 (s, 2 H), 5.20 (s, 4 H). ^{13}C NMR (CDCl₃): δ 184.1, 145.2, 136.1, 134.6, 128.5, 128.4, 128.1, 75.1. Anal. Calcd for C₂₀H₁₆O₄: C, 74.99; H, 5.03. Found: C, 75.04; H, 5.06. HRMS (FAB) calcd for C₂₀H₁₆O₄ (M + H⁺): 320.1049. Found: 320.1059.

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40 2,3,4-Tribenzyloxybenzaldehyde 10. Kolonits, P. et al., *Acta Chim. Hung.* 113:367 (1983). POCl₃ (155 mL, 1.66 mol) was slowly added to N-methylformanilide (175 mL, 1.4 mol) at room temperature under Ar which resulted in formation of a yellow solid. After 2 h, the solid was treated with a solution of 1,2,3-tribenzyloxybenzene 8 (20 g, 51 mmol) in anhydrous DMF (40 mL) and

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temperature and then poured into ice water (3 L) with vigorous stirring for 12 h. The resulting brown precipitate was filtered, washed with hexanes (3 x 100 mL) and finally recrystallized from MeOH to afford **10** (19.8 g, 93%) as a white powder. mp 73 - 74 °C. ^1H NMR (CDCl_3): δ 10.11 (s, 1 H), 7.57 (d, J = 9 Hz, 1 H), 7.44-7.28 (m, 15 H), 6.83 (d, J = 9 Hz, 1 H), 5.21 (s, 2 H), 5.16 (s, 2 H), 5.08 (s, 2 H); ^{13}C NMR (CDCl_3): δ 188.8, 158.5, 155.9, 141.1, 136.9, 136.2, 135.8, 128.6, 128.5 (2), 128.3 (2), 128.2, 127.5, 124.0, 109.1, 76.8, 75.5, 70.9. Anal. Calcd for $\text{C}_{28}\text{H}_{24}\text{O}_4$: C, 79.22; H, 5.70. Found: C, 79.17; H, 5.80. HRMS (FAB) calcd for $\text{C}_{28}\text{H}_{24}\text{O}_4$ ($M + \text{H}^+$): 424.1675. Found: 424.1669.

2,3,4-Tribenzyloxyphenol **11**. Kolenits, P. et al., *Acta Chim Hung.* 113:367 (1983). A solution of 30% H_2O_2 (6 mL, 57.8 mmol) and 85% formic acid (32 mL, 600 mmol) was added dropwise to a solution of 2,3,4-tribenzyloxybenzaldehyde **10** (9.8 g, 23.1 mmol) in CH_2Cl_2 (50 mL) over 30 min at 0 °C. After 1 h of stirring at 0 °C, the reaction was stirred at room temperature for 24 h. The reaction was subsequently cooled to 4 °C and diluted with 10% (w/v) aqueous Na_2SO_3 (50 mL). The aqueous phase was washed with CH_2Cl_2 (3 x 40 mL). Drying and concentration afforded a brown oil which was dissolved in a methanolic solution of NaOMe (30 mL, 0.1 N) and refluxed. After 10 min, the solution was cooled to 4 °C and acidified with 6 N HCl. MeOH was removed *in vacuo*. The mixture was diluted with H_2O (15 mL) followed by extraction of the aqueous phase with benzene (3 x 40 mL). Drying and concentration afforded **11** (9.0 g, 95%) as a brown oil. ^1H NMR (CDCl_3): δ 7.45 - 7.31 (m, 15 H), 6.65 (d, J = 9 Hz, 1 H), 6.58 (d, J = 9 Hz, 1 H), 5.28 (s, 1 H), 5.12 (s, 2 H), 5.11 (s, 2 H), 5.04 (s, 2 H). ^{13}C NMR (CDCl_3): δ 146.0, 144.0, 142.0, 139.6, 137.3, 137.1, 136.8, 128.4, 128.3 (2), 128.2, 127.9, 127.7, 127.4, 110.4, 109.0, 75.6; 75.3, 71.7. Anal. Calcd for $\text{C}_{27}\text{H}_{24}\text{O}_4$: C, 78.62; H, 5.87. Found: C, 78.71; H, 5.86. HRMS (FAB) calcd for $\text{C}_{27}\text{H}_{24}\text{O}_4$ ($M + \text{H}^+$): 412.1675. Found: 412.1673.

SPECIFIC EXAMPLE 3

Synthesis Of Coenzyme Q From 1,2,3,4-Tetrahydroxybenzene

30 I. Results

Variations in strategies employed for hydroxyl protection combined with the ease of metallation and alkylation of the aromatic nucleus makes **1** a versatile intermediate for the synthesis of a wide spectrum of naturally-occurring 1,2,3,4-tetrahydroxybenzene derivatives. For example, permethylation (Figure 5) of **1** leads to tetramethyl **12** which undergoes facile lithiation and methylation affording **13** in

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high yield. Formation of an organocuprate from 13, farnesylation, and subsequent reaction with $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$ affords coenzyme Q_{n+3} , 4. This four-step synthesis of coenzyme Q_n from tetrahydroxybenzene, 1 is equal in length to the shortest reported (Keinan, E. et al., *J. Org. Chem.* 52:3872 (1987)) synthesis of coenzyme Q_n which uses *p*-cresol as a starting material and substantially shorter than syntheses of coenzyme Q_n from pyrogallol, gallic acid, or vanillin. Syper, L. et al., *Tetrahedron* 36:123 (1980); Sugihara, H. et al., *Liebigs Ann. Chem.* 763:109 (1972); Mayer, J. et al., *Meth. Enzymol.* 18:182 (1971).

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Only one oxygen atom in coenzyme Q_n , a shikimate pathway product, is directly derived from D-glucose. The remaining oxygen atoms are derived from O_2 via enzyme-catalyzed hydroxylations. Trihydroxybenzenes, pyrogallol, and phloroglucinol possess the maximum number of oxygen atoms attached to a benzene nucleus by the shikimate pathway or polyketide biosynthesis in lieu of enzyme-catalyzed hydroxylation. At least a dozen enzymes are required to disassemble and reassemble the carbon atoms of D-glucose into the benzene nucleus of coenzyme Q_n , pyrogallols, and phloroglucinols. By comparison, synthesis of 1,2,3,4-tetrahydroxybenzene, 1 via *myo*-inositol intermediacy requires only four enzymes and an acid-catalyzed dehydration for all six carbon and all four oxygen atoms to be directly derived from the carbon and oxygen atoms of D-glucose. The method of the present invention for synthesis of 1,2,3,4-tetrahydroxybenzene, 1 is thus a useful example of enzyme and atom (Trost, B.M., In *Green Chemistry*, Anastas, P.T., Williamson, T.C. Eds., Oxford: New York, Chap. 6 (1998)) economy in organic synthesis in addition to being a significant strategic departure from previous biocatalytic syntheses of aromatic chemicals from D-glucose.

II. Materials And Methods

General. ^1H NMR spectra were recorded on a 300 MHz spectrometer.

Chemical shifts for ^1H NMR spectra are reported (in parts per million) relative to internal tetramethylsilane (Me_4Si , $\delta = 0.0$ ppm) with CDCl_3 as solvent, to sodium

30 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (TSP, $\delta = 0.0$ ppm) when D_2O was the solvent, and to acetone (CH_3COCD_3 , $\delta = 2.04$ ppm) with d_6 -acetone. ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts for ^{13}C NMR spectra are reported (in parts per million) relative to CDCl_3 ($\delta = 77.0$ ppm), relative to CD_3COCD_3 ($\delta = 29.8$ ppm), and relative to internal CH_3OH ($\delta = 49.0$ ppm) or 35 internal CH_3CN ($\delta = 1.4$ ppm) in D_2O . FAB mass spectra were performed by

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University of South Carolina (Columbia, SC). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

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Radial chromatography was carried out with a Harrison Associates

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5 Chromatotron using 1, 2 or 4 mm layers of silica gel 60 PF₂₅₄ containing gypsum (E. Merck). Silica gel 60 (40 - 63 μm E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, E. Merck or Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) followed by heating. Dimethylformamide, N-methylformanilide and acetone were dried and stored over activated Linde 4 Å molecular sieves under Ar. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. n-Hexanes and TMEDA were distilled from sodium under Ar and stored over activated Linde 4 Å molecular sieves under Ar. Organic solutions of products were dried over MgSO₄.

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1,2,3,4-Tetramethoxybenzene 12. Syper, L. et al., *Tetrahedron* 36:123 (1980). A solution of 1,2,3,4-tetrahydroxybenzene 1 (8.4 g, 59 mmol) and dimethyl sulfate (37.5 mL, 0.396 mol) in EtOH (21 mL) was added dropwise to an 8.5 M aqueous solution of NaOH (42 mL) over 20 min at room temperature. After

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2 h, the reaction was diluted with H₂O (300 mL) and cooled to -20 °C for 12 h. The resulting precipitate was filtered, washed with H₂O, and then recrystallized from hexanes to afford 12 (8.12 g, 69%) as colorless needles. mp 84 - 85 °C. ¹H NMR (CDCl₃): δ 6.58 (s, 2 H), 3.90 (s, 6 H) 3.82 (s, 6 H). ¹³C NMR (CDCl₃): δ 147.7, 143.3, 106.3, 61.1, 56.3. Anal. Calcd for C₁₀H₁₄O₄: C, 60.59; H, 7.12.

25 Found: C, 60.44; H, 7.07.

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2,3,4,5-Tetramethoxytoluene 13. Syper, L. et al., *Tetrahedron* 36:123 (1980). To a solution of 1,2,3,4-tetramethoxybenzene 12 (4.0 g, 20.2 mmol) and TMEDA (6 mL, 38.0 mmol) in hexanes (44 mL) and THF (80 mL) at 0 °C under Ar, n-BuLi in hexane (1.6 M, 25.6 mmol) was added dropwise over a 10 min period and 30 the reaction stirred for 30 min at 0 °C under Ar. Subsequent to dropwise addition of CH₃I (20 mL, 160 mmol) over an 8 min period, the reaction was stirred for 3 h at 0 °C under Ar and then quenched by addition of aqueous NH₄Cl and ether (20 mL). The organic layer was sequentially washed with concentrated NH₄OH, water, and brine. Drying and concentration of the organic layer was followed by 35 purification of the residue by flash chromatography (hexanes, hexanes/EtOAc, 19:1,

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v/v) to afford 13 as a clear oil (3.6 g, 83%). ^1H NMR (CDCl_3): δ 6.45 (s, 1 H), 3.93 (s, 3 H), 3.87 (s, 3 H), 3.82 (s, 3 H), 3.79 (s, 3 H), 2.23 (s, 3 H). ^{13}C NMR (CDCl_3): δ 149.0, 146.9, 145.3, 140.7, 125.7, 108.2, 61.0, 60.9, 60.5, 55.9, 15.7.

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Protected coenzyme Q₃, 14. Keinan, E. et al., *J. Org. Chem.* 52:3872

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5 (1987). *n*-BuLi (1.6 M, 0.9 mL) was added dropwise over a 15 min period to a solution of 1,2,3,4-tetramethoxytoluene 13 (0.212 g, 1 mmol) and TMEDA (0.3 mL, 1.9 mmol) in hexane (2.2 mL) at 0 °C under Ar. This yellow precipitate-containing reaction mixture was then stirred at 0 °C under Ar for 30 min, diluted with THF (4 mL) and ether (11 mL), followed by addition of CuCN (0.125 g, 1.4 mmol). After stirring for 30 min at 0 °C under Ar, the temperature was reduced to -78 °C, and a solution of farnesyl bromide (0.286 g, 1 mmol) in hexane (2 mL) was dropwise added over a 30 min period. Further reaction for 3 h at -78 °C and subsequent slow warming to room temperature was followed by addition of saturated aqueous NH_4Cl (10 mL) and ether (20 mL). Washing the organic phase with concentrated NH_4OH , water, and brine was followed by drying and concentration. Purification of the residue by radial chromatography (2 mm thickness, hexane/EtOAc, 9:1, v/v) afforded 14 as a clear oil (0.236 g, 57%). ^1H NMR (CDCl_3): δ 5.12-5.01 (m, 3 H), 3.90 (s, 6 H), 3.78 (s, 6 H), 3.32 (d, J = 7 Hz, 2 H), 2.14 (s, 3 H), 2.08-1.91 (m, 8 H), 1.77 (s, 3 H), 1.66 (s, 3 H), 1.58 (s, 6 H). ^{13}C NMR (CDCl_3): δ 147.8, 147.6, 144.9, 144.6, 135.0, 134.9, 131.2, 129.2, 125.4, 124.3, 124.1, 122.8, 61.1, 60.6, 39.7, 26.7, 26.5, 25.7, 25.6, 17.6, 16.2, 15.9, 11.7.

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Coenzyme Q₃, 4. Keinan, E. et al., *J. Org. Chem.* 52:3872 (1987). A suspension maintained at 0 °C resulting from addition of pyridine-2,6-dicarboxylate (0.125 g, 0.75 mmol) to a solution of protected coenzyme Q₃ 14 in CH_3CN (1.4 mL) and water (0.6 mL) at 0 °C was reacted with a 0 °C solution of $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$ (0.411 g, 0.75 mmol) in CH_3CN (0.4 mL) and water (0.4 mL) added dropwise over a 10 min period. After 40 min at 0 °C, the reaction was warmed to room temperature and stirred for 20 min. Water (10 mL) was added to the reaction mixture and the resulting solution extracted with CH_2Cl_2 (3x 100 mL). The combined organic phases were dried, concentrated, and purified by radial chromatography (1 mm thickness, hexane/EtOAc, 19:1, v/v) to afford 4 (0.053 g, 46%) as an orange oil. ^1H NMR (CDCl_3): δ 5.07 (dd, J = 7, 7 Hz, 1 H), 5.05 (dd, J = 7, 7 Hz, 1 H), 4.94 (dd, J = 7, 7 Hz, 1 H), 3.99 (s, 3 H), 3.98 (s, 3 H), 3.18 (d, J = 6.8 Hz, 2 H), 2.08-1.91 (m, 8 H), 2.01 (s, 3 H), 1.74 (s, 3 H), 1.67 (s, 3

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H), 1.59 (s, 3 H), 1.58 (s, 3 H); ^{13}C NMR (CDCl_3): δ 184.7, 183.9, 144.3, 144.2, 141.6, 138.8, 137.6, 135.2, 131.3, 124.3, 123.8, 118.8, 61.1, 39.7, 26.7, 26.4, 25.7, 25.3, 17.6, 16.3, 16.0, 11.9; HRMS (FAB) calcd for $\text{C}_{24}\text{H}_{34}\text{O}_4$ ($\text{M} + \text{H}^+$): 386.2457. Found: 386.2461

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SPECIFIC EXAMPLE 4**Synthesis Of Myo-2-Inosose By A Single Microbe**

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Myo-2-inosose (1 g/L), *myo*-inositol (18 g/L), and *myo*-inositol-1-phosphate (3.1 g/L) were synthesized by *E. coli* JWF1/pAD2.28A in 9.6% (mol/mol) yield from glucose under fed-batch fermentation conditions as described in Specific Example 10. The fermentation ran for 54 h with incremental addition of IPTG (0.0048 g added each time) at 7 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, and 54 h. The *myo*-2-inosose can then be converted to 1,2,3,4-tetrahydroxybenzene by acid-catalyzed dehydration as described in Specific Example 1.

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Plasmid pAD2.28A carries *INO1* under P_{lac} promoter control, *io/G* under P_{lac} promoter control, *lacZ*, and *serA*. *INO1* encodes *myo*-inositol-1-phosphate synthase and was cloned out of *Saccharomyces cerevisiae*. Inositol dehydrogenase is encoded by the *io/G* locus, which was cloned out of *Bacillus subtilis*.

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SPECIFIC EXAMPLE 5**Conversion Of 1,2,3,4-Tetrahydroxybenzene To Pyrogallol**

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20 A solution consisting of 0.4 g of NaOH (10 mmol) dissolved in 10 mL H_2O was freeze-thaw degassed three times under Ar. This solution was then added via cannula under Ar to a 250 mL Parr bottle containing 1,2,3,4-tetrahydroxybenzene (1.42 g, 10 mmol) and 5% Rh/ Al_2O_3 (0.25 g), which had been flushed with Ar and then sealed with a septum. The resulting red/brown solution was hydrogenated 25 under 50 psi H_2 using a Parr Hydrogenator. After 12 h, the solution was filtered through Celite® and the catalyst rinsed with 10 mL H_2O . The resulting dark brown solution was adjusted to pH = 6.0 with 10% HCl and then concentrated to a brown oil. The oil was dissolved in 50 mL 0.5 M H_2SO_4 that had been degassed by aeration with Ar for 20 minutes. The solution was heated to reflux under Ar for 40 30 12 h, cooled to room temperature, and then extracted with Et_2O (4 x 50 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated to a brown oil. Kugel-Rohr distillation of the oil under vacuum (1 mm Hg) at 90 °C afforded 0.56 g pyrogallol (4.44 mmol, 44% yield) as a white, crystalline solid.

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50 The foregoing discussion discloses and describes merely exemplary 35 embodiments of the present invention. One skilled in the art will readily recognize

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from such discussion, and from the accompanying drawings and claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

5 All references cited herein are incorporated by reference as if fully set forth.

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WE CLAIM:

1. A method for the production of 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source, comprising the steps of:
 - 10 a) converting the carbon source to *myo*-inositol with a first recombinant microbe;
 - 5 b) converting the *myo*-inositol to *myo*-2-inosose with a second microbe; and
 - 15 c) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.
- 20 2. The method of Claim 1, wherein the first recombinant microbe comprises the gene encoding *myo*-inositol-1-phosphate synthase.
- 25 3. The method of Claim 2, wherein the gene encoding *myo*-inositol-1-phosphate synthase is a *S. cerevisiae INO1* gene.
- 30 4. The method of Claim 3, wherein the *S. cerevisiae INO1* gene is located on a plasmid in the recombinant microbe.
- 35 5. The method of Claim 4, wherein the plasmid is pAD1.88A.
- 40 6. The method of Claim 1, wherein the first recombinant microbe is *E. coli* JWF1/pAD1.88A.
- 45 7. The method of Claim 1, wherein the second microbe expresses inositol dehydrogenase activity.
- 50 8. The method of Claim 1, wherein the second microbe is *Gluconobacter oxydans*.
- 55 9. The method of Claim 8, wherein the *Gluconobacter oxydans* is *Gluconobacter oxydans* ATCC 621.
- 25 10. The method of Claim 1, wherein the second microbe is a recombinant microbe comprising the gene for inositol dehydrogenase.

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11. The method of Claim 10, wherein the gene for inositol dehydrogenase is a *Bacillus subtilis iolG* gene.

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12. The method of Claim 1, wherein the carbon source is glucose.

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13. The method of Claim 1, further comprising the step of reducing the 5 1,2,3-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

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14. A method of producing 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source comprising the steps of:

a) converting the carbon source to *myo*-inositol with a recombinant *E. coli* comprising the gene encoding *myo*-inositol-1-phosphate synthase;

10 b) converting the *myo*-inositol to *myo*-2-inosose with a microbe that expresses inositol dehydrogenase activity; and

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c) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

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15. The method of Claim 14, wherein the gene encoding *myo*-inositol-1-phosphate synthase is the *S. cerevisiae INO1* gene.

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16. The method of Claim 15, wherein the *INO1* gene is located on a plasmid in the recombinant *E. coli*.

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17. The method of Claim 16, wherein the plasmid is pAD1.88A.

18. The method of Claim 14, wherein the recombinant *E. coli* is *E. coli* 20 JWF1/pAD1.88A.

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19. The method of Claim 14, wherein the carbon source is glucose.

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20. A method for the production of 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source, comprising the steps of:

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- a) converting the carbon source to *myo*-2-inosose with a recombinant microbe; and
- 5 b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

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21. The method of Claim 20, wherein the recombinant microbe comprises the genes encoding for *myo*-inositol-1-phosphate synthase and inositol dehydrogenase.

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10 22. The method of Claim 21, wherein the gene encoding for *myo*-inositol-1-phosphate synthase is a *Saccharomyces cerevisiae* INO1 gene.

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23. The method of Claim 22, wherein the INO1 gene is located on a plasmid in the recombinant microbe.

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24. The method of Claim 23, wherein the plasmid is pAD2.28A.

15 25. The method of Claim 21, wherein the gene encoding for inositol dehydrogenase is an *ioIG* gene.

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26. The method of Claim 25, wherein the *ioIG* gene is a *Bacillus subtilis* *ioIG* gene.

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27. The method of Claim 25, wherein the *ioIG* gene is located on a 20 plasmid in the recombinant microbe.

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28. The method of Claim 27, wherein the plasmid is pAD2.28A.

29. The method of Claim 20, wherein the recombinant microbe is JWF1/pAD2.28A.

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30. A method of producing 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source comprising the steps of:
10 a) converting the carbon source to *myo*-2-inosose with a recombinant *E. coli* comprising the genes encoding for *myo*-1-phosphate synthase and inositol
5 dehydrogenase; and
15 b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

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31. The method of Claim 30, wherein the gene encoding for *myo*-inositol-1-phosphate synthase is the *S. cerevisiae INO1* gene.

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10 32. The method of Claim 30, wherein the gene encoding for inositol
dehydrogenase is the *Bacillus subtilis ioG* gene.

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33. The method of Claim 30, wherein the recombinant *E. coli* is JWF1/pAD2.28A.

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34. A method for the production of 1,2,3-trihydroxybenzene and derivatives thereof from a carbon source, comprising the steps of:
15 a) converting the carbon source to *myo*-inositol with a first recombinant
microbe;
35 b) converting the *myo*-inositol to *myo*-2-inosose with a second microbe;
c) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid
20 catalyzed dehydration; and
40 d) reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

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35. The method of Claim 34, wherein the first recombinant microbe comprises the gene encoding *myo*-inositol-1-phosphate synthase.

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25 36. The method of Claim 35, wherein the gene encoding *myo*-inositol-1-phosphate synthase is the *S. cerevisiae INO1* gene.

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37. The method of Claim 36, wherein the *S. cerevisiae INO1* gene is located on a plasmid in the recombinant microbe.

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38. The method of Claim 37, wherein the plasmid is pAD1.88A.

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39. The method of Claim 34, wherein the first recombinant microbe is *E. coli* JWF1/pAD1.88A.

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40. The method of Claim 34, wherein the second microbe expresses
5 inositol dehydrogenase activity.

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41. The method of Claim 34, wherein the second microbe is
Gluconobacter oxydans.

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42. The method of Claim 41, wherein the *Gluconobacter oxydans* is
Gluconobacter oxydans ATCC 621.

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10 43. The method of Claim 34, wherein the carbon source is glucose.

44. The method of Claim 34, wherein the 1,2,3,4-tetrahydroxybenzene
is reduced to 1,2,3-trihydroxybenzene by catalytic hydrogenation and acid catalyzed
hydrolysis.

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45. The method of Claim 44, wherein the catalytic hydrogenation uses
15 Rh/Al₂O₃ as the catalyst.

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46. A method for the production of 1,2,3-trihydroxybenzene and
derivatives thereof from a carbon source, comprising the steps of:

a) converting the carbon source to *myo*-2-inosose with a recombinant
microbe;

20 b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid
catalyzed dehydration; and

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c) reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-
trihydroxybenzene.

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47. The method of Claim 46, wherein the recombinant microbe comprises
25 the genes encoding for *myo*-inositol-1-phosphate synthase and inositol
dehydrogenase.

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48. The method of Claim 47, wherein the gene encoding for *myo*-inositol-1-phosphate synthase is a *Saccharomyces cerevisiae* INO1 gene.

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49. The method of Claim 48, wherein the INO1 gene is located on a plasmid in the recombinant microbe.

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50. The method of Claim 49, wherein the plasmid is pAD2.28A.

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51. The method of Claim 47, wherein the gene encoding for inositol dehydrogenase is an *io/G* gene.

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52. The method of Claim 51, wherein the *io/G* gene is a *Bacillus subtilis* *io/G* gene.

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53. The method of Claim 51, wherein the *io/G* gene is located on a plasmid in the recombinant microbe.

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54. The method of Claim 53, wherein the plasmid is pAD2.28A.

55. The method of Claim 46, wherein the recombinant microbe is JWF1/pAD2.28A.

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56. The method of Claim 34, wherein the 1,2,3,4-tetrahydroxybenzene is converted to 1,2,3-trihydroxybenzene by catalytic hydrogenation and acid catalyzed hydrolysis.

57. The method of Claim 56, wherein the catalytic hydrogenation uses Rh/Al₂O₃ as the catalyst.

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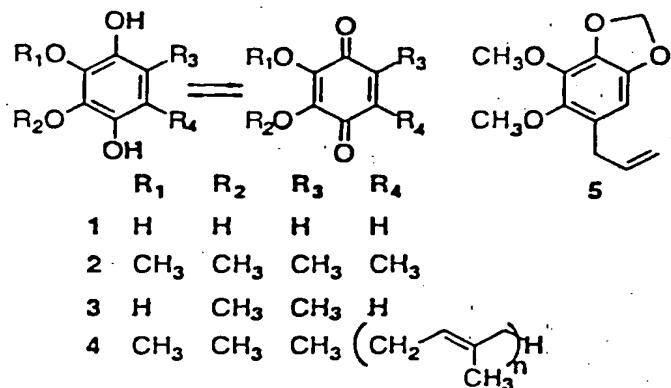
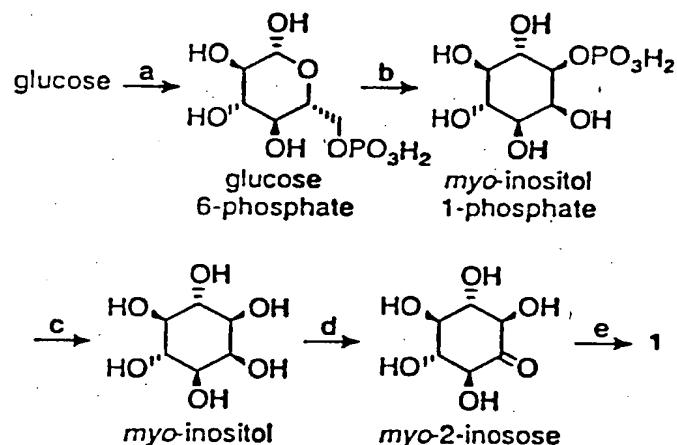


FIGURE 1



^aKey: (a) phosphoenolpyruvate:carbohydrate phosphotransferase; (b) *myo*-inositol 1-phosphate synthase; (c) phosphatase activity; (d) dehydrogenase activity; (e) 0.5 M H₂SO₄, H₂O, reflux.

FIGURE 2

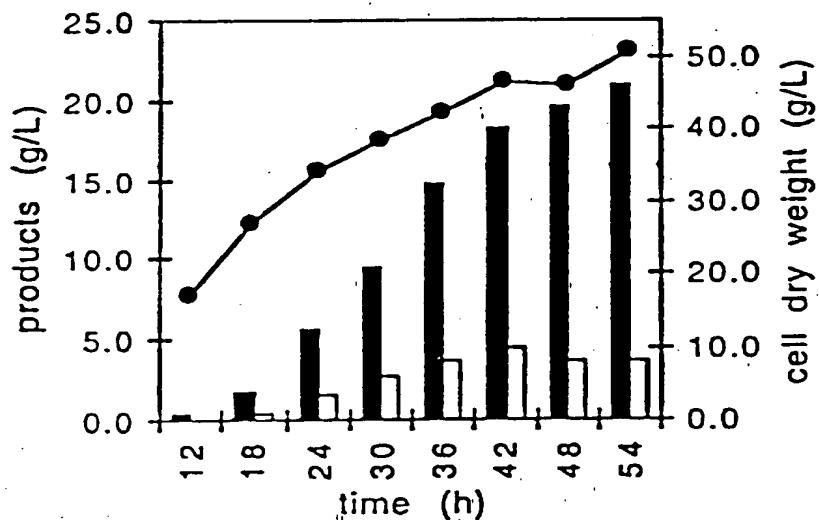
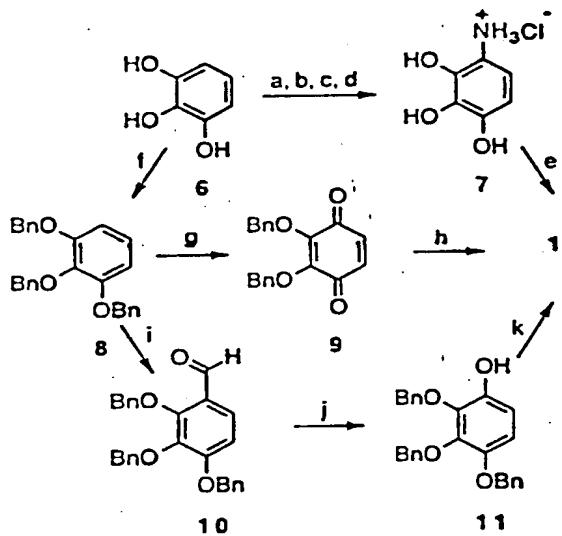


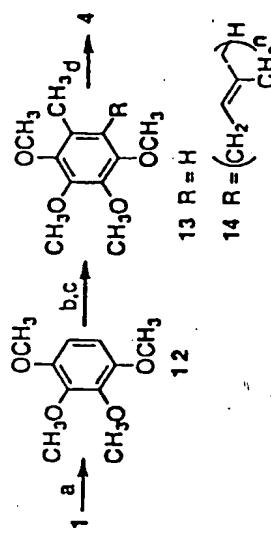
FIGURE 3

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^aKey: (a) $\text{Cl}_2\text{C}(\text{O})$, pyridine, xylene, reflux; (b) H_2SO_4 , HNO_3 ; (c) KOH (aq.); (d) Zn , HCl ; (e) H_2O , reflux; (f) BnBr , K_2CO_3 , acetone, reflux, 83 %; (g) $\text{K}_3\text{Fe}(\text{CN})_6$, H_2O_2 , AcOH , 11 %; (h) H_2 , 10 % Pd/C , EtOH , 100 %; (i) N -methylformanilide, POCl_3 , 60 °C, 93 %; (j) HCO_2H , H_2O_2 , CH_2Cl_2 , 0 °C to rt, 95 %; (k) H_2 , 10 % Pd/C , EtOH , 80%.

FIGURE 4



^aKey: (a) $(\text{CH}_3)_2\text{SO}_4$, NaOH , 69 %; (b) (i) $n\text{-BuLi}$, TMEDA, hexanes, THF, 0 °C; (ii) CH_3I , 0 °C, 83%; (c) (i) $n\text{-BuLi}$, TMEDA, hexanes, 0 °C; (ii) CuCN , THF, Et_2O , 0 °C; (iii) famesyl bromide, -78 °C, 57%; (d) CAN, pyridine-2,6-dicarboxylate, $\text{CH}_3\text{CNH}_2\text{O}$, 0 °C, 46%.

FIGURE 5

INTERNATIONAL SEARCH REPORT

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|---|
| International application No. PCT/US00/06808 |
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| A. CLASSIFICATION OF SUBJECT MATTER | | |
| IPC(7): C12P 7/00, 39/00 US CL : 435/132, 42 According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/132, 42 | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN, search terms: 642-96-6 (Registry No. for 1,2,3,4-tetrahydroxybenzene), inositol, inosose, dehydrogenase, synthase, metabolism, microorganism, yeast, review | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X, P --- | HANSEN et al. Synthesis of 1,2,3,4-Tetrahydroxybenzene from D-Glucose. Journal of the American Chemical Society. 21 April 1999, Vol. 121, pages 3799-3800, see entire document. | 1-10, 12, 14-23, 30-31 |
| Y, P | | ----- 11,13,24-29, 32- 57 |
| Y | YOSHIDA et al. Organization and Transcription of the myo-Inositol Operon, <i>iol</i> , of <i>Bacillus subtilis</i> . Journal of Bacteriology. July 1997, Vol. 179, No. 14, pages 4591-4598, see entire document. | 11, 24-29, 32-33, 50-55 |
| Y | DEAN-JOHNSON et al. Biosynthesis of Inositol in Yeast. The Journal of Biological Chemistry. 15 January 1989, Vol. 264, No. 2, pages 1274-1283, see entire document. | 3-6, 15-18, 22-24, 29, 31, 33, 36-39, 48-50, 54-55 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| * Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed | | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *A* document member of the same patent family |
| Date of the actual completion of the international search 12 JUNE 2000 | Date of mailing of the international search report 05 JUL 2000 | |
| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | Authorized officer KATHLEEN KERR Telephone No. (703) 308-0190 | |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06808

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|--|-----------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | WHITE et al. Inositol Metabolism in Yeasts. Advances in Microbial Physiology. London: Academic Press, 1991, Vol. 32, pages 1-51. | 1-57 |

Form PCT/ISA/210 (continuation of second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06808

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06808

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-33, drawn to methods for the production of 1,2,3,4-tetrahydroxybenzene.

Group II, claims 34-57, drawn to methods for the production of 1,2,3-trihydroxybenzene.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is the methods for producing 1,2,3,4-tetrahydroxybenzene via myo-inositol and myo-2-inosine in recombinant microbes. This special technical feature does not relate to the same inventive concept as the methods for producing a distinct compound, namely 1,2,3-trihydroxybenzene, although some of the microbes used can be identical between the two inventive concepts.

Although the Examiner finds a lack of unity between the two Groups, the Examiner will examine both Groups in the search report because the search for each of the Groups is somewhat overlapping. Thus, the invention of the methods for producing 1,2,3,4-tetrahydroxybenzene and the invention of the methods for producing 1,2,3-trihydroxybenzene have been searched. The Examiner reserves the right to uphold this lack of unity in the Chapter 2 phase of this PCT case.

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- OTHER:** _____

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